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Molecular Basis of Pathogenicity in Enteric Bacteria

Annual and Final Report

Stanley Falkow, Ph.D.

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A new quantitative assay for the determination of the E. coli heat-stable enterotoxin has been developed which is about 10-fold more sensitive than existing methods. A strategy for the development of subunit vaccines has been tested using specific peptides of the binding region of cholera toxin. Although there have been numerous reports of the relationship between E. coli LT toxin (and cholera toxin) and a putative enterotoxin of Salmonella typhimurium, we have been unable to demonstrate any DNA sequence homology between known toxin sequences of E. coli LT, ST or cholera toxin with the nucleic acid of Salmonella.			
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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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TABLE OF CONTENTS

Preface.....	2
Structure and Function of the Cholera Toxin B Subunit.....	3
Table 1. Binding Characteristics of Holme's Monoclonal Anti-CT Antibodies.....	6
Characterization of a <u>Salmonella typhimurium</u> Adhesin.....	7
Table 2. Distribution of MRHA Among Salmonella Strains.....	8
Genetics and Molecular Biology of Genes of Shigellae and Yersiniae which Contribute to Eucaryotic Cell Invasion and Subsequent Multiplication.....	11
Table 3. Behavior of <u>E. coli</u> Carrying Cloned <u>Y. pseudotuberculosis</u> DNA.....	15
References Cited.....	17
Figure 1. Predicted Secondary Structure of LT-B Polypeptide.....	18
Figure 2. Average Hydropathicity of St-P Polypeptide Calculated According to the Method of Hopp and Woods.....	20
Figure 3. Kinetics of Adsorption to HEp-2 Cells by <u>Y. pseudotuberculosis</u> and <u>E. coli</u> Containing the Plasmid that Confers Invasion.....	22

PREFACE

Our research progress is presented in several sections, representing several diverse areas of effort. The major research participants supported completely, or in part, by contract monies are mostly graduate students who are Ph.D. candidates in the Department of Medical Microbiology mentored by me, or in conjunction with Dr. B.A.D. Stocker or Dr. Gary Schoolnik. The work itself spans such subjects as the functional basis of enterotoxin structure, the first molecular characterization of an adhesin of Salmonella typhimurium, as well as the first cloning of a relatively small genetic sequence that confers the capacity upon E. coli K-12 to invade human tissue culture cells. This latter work, in particular, will form the basis for our proposal for continuing support.

1. Structure and Function of the Cholera toxin B subunit.

1.A Plan of Study. Last year we proposed studies to dissect the molecular structure of cholera toxin and suggested a theoretical general strategy by which subunit vaccines might be directly synthesized in the laboratory. This investigation was initiated not only to serve as a potential novel approach to vaccine development but also as a means to understand better the functional domains of bacterial toxins and their biological action.

Cholera toxin is an 86,000 dalton protein composed of three polypeptide chains A1, A2 and B with molecular weights of 22,000, 5,000 and 11,600, respectively. A disulfide-bridge links the A1 and A2 to form toxin subunit A (CT-A) which associates non-covalently with a pentameric aggregate of B-chains, subunit B (CT-B). The amino acid sequences are available as are the sequences of the closely related heatlabile enterotoxin (LT) of *E. coli*; some of these latter sequences were developed in our laboratory under previous contract support (1,2).

The molecular basis for the biological effects of CT and LT have been investigated extensively and are well known: toxin liberated in the small bowel lumen by toxigenic microorganisms bind to specific epithelial cell receptors. In particular, the B-subunit associates with the oligosaccharide moiety of the plasma membrane monosialoganglioside, GM-1. Subsequently, subunit A enters the plasma membrane and catalyzes the ADP-ribosylation of a GTP-binding protein, resulting in the activation of adenylate cyclase. The ensuing increase in intracellular levels of cAMP provokes hypersecretion of chloride, bicarbonate and water.

As we noted last year, while the sequence of events initiated by CT and LT have been elucidated in some detail, the precise basis by which the polypeptide chain exerts its effects remains undefined. Specifically, the molecular determinants which serve to bind the toxin to its receptor remain largely unknown. Recognizing the importance of this system as a model of receptor-ligand interactions in general, and of carbohydrate-protein binding, in particular, we chose this aspect of the molecular definition of CT and LT upon which to initially concentrate and our first experiments, which were reported in Annual Report dated October 1983, dealt with this aspect of the work. The specific objectives of this research are:

1. To explore the antigenicity of LT-B and CT-B and create a topological map locating the relative and absolute positions of antibody combining sites.
2. To identify amino acid residues which mediate receptor recognition.
3. To conduct structure function studies - using mutant strains and receptor analogues - which seek to elucidate the molecular geometry of the binding event.

These objectives have been, or will be, approached as follows. First, as reported last year, and now extended materially, a peptide "library" has been constructed. Each entry in the library, containing synthetic analogues and proteolytic fragments, has been examined for receptor binding function. In addition, anti-peptide antibodies have been raised and their properties assessed. Second, monoclonal antibodies were obtained from Professor Randall Holmes of the Uniformed Services University for the Health Sciences by the primary investigator of this subproject, David Ludwig, an M.D.-Ph.D candidate, these have been characterized and have been found useful in defining functionally important amino acid residues.

1.8 Results and Discussion. Work on this project began in the latter part of 1982. Last year we described the synthesis of two synthetic peptides corresponding to predicted regions of importance of CT-8. Subsequently, we have prepared nine additional synthetic peptides. Peptides were selected for synthesis based on several lines of evidence. The amino acid sequence was subjected to the hydrophilicity analysis of Hopp and Woods (3) and is shown in Figure 1. Areas of greatest hydrophilicity, for example amino acid residues 30-37, 60-71, and 79-84, were predicted to reside in external portions of the protein, and constituted attractive candidates for synthetic immunogens. Using the predictive rules of Chou and Fasman (4) the relative probabilities of alpha-helix, beta-sheet, and beta-turn, were calculated and a model of secondary domains were proposed as illustrated in Figure 2. Peptides were selected in an attempt to preserve these structural features, Beta-turns in particular. Preliminary studies by Holmgren, Kohn and others (5,6) also suggested several specific amino acid residues which resided within the receptor binding domain.

Ten peptides and one heterodimer have been synthesized. Cysteine, where possible, was placed at the N or C terminus of each peptide to facilitate coupling to carrier proteins. The peptides synthesized were:

SP1:	NH ₂ - CKKAIERMKNTLR	- COOH	(residues 62-73)
SP2:	VEVPGSQHIDSQC		50-61
SP3:	CVWNNTPHAI		86-97
SP4:	CSYTESLAGKREMA		26-38
SP5:	CAEYHNTQIHTLN		9-21
SP6:	CITFKNGATFQV		40-50
SP7:	TPQNITDLC		1-9
SP8:	RIAAYLTEAKVEKLC		73-86
SP9:	EAKVEKLCVWNNKT		79-92
SP10:	TPQNITDLCAEYHNTQ		1-16
SP11:	EAKVEKLCVWNNKT		
	S-S		
	TPQNITDLCAEYHNTQ		

Peptide synthesis was performed by the Merrifield technique (7) and the completed peptide simultaneously deprotected and cleaved from the

solid support by hydrolysis with hydrogen flouride. Purity of the peptides was assessed by reverse phase-high pressure liquid chromatography (RP-HPLC). In addition to the synthesized peptides, the three major fragments produced by CNBr cleavage were purified by RP-HPLC. These are CNBr-1, amino acid residues 1-37; CNBr-2, residues 38-68; and CNBr-3 residues 69-101.

Initially, we examined the functional properties of SP1-S:P11 and CNBr1-CNBr-3. The antigenicity of these peptides and fragments were assessed by Radio Immune Assay (RIA) for crossreactivity with polyclonal antisera to CT-B. Only SP6 showed significant binding. This peptide is derived from a region of CT-B which differs slightly from human LT-B and markedly from porcine LT-B (W.S. Dallas, personal communication) and may constitute a "strain-specific" antigenic determinant. The ability of SP1-SP8 to bind GM-1 was examined in competition studies (with non-radioactive peptide and ^{125}I CT-B). No detectable affinity could be demonstrated. This result, while disappointing, was not surprising and had been anticipated in our proposal. By analogy to other sugar-binding proteins, the receptor binding domain of CT-B is probably composed of non-contiguous amino acid residues critically arrayed in a conformation complementary to the GM1 oligosaccharide. Therefore anti-peptide antibodies were raised in rabbits in the hope that these reagents, by virtue of predetermined specificity, would provide indirect information regarding the molecular requirements of receptor recognition.

The anti-peptide antibodies were prepared by the systemic immunization of rabbits with peptides conjugated to protein carriers. Briefly, the heterobifunctional crosslinker SMCC was added to BSA and the mixture subsequently desalted over a G25 column. The peptide, reduced with NaBH_4 , was added and the mixture incubated overnight. The resulting protein-peptide conjugate was mixed with complete Freund's adjuvant and administered in multiple intramuscular injections. Booster immunizations were given in incomplete Freund's Adjuvant at monthly intervals and sera assessed by RIA.

While vigorous anti-peptide responses were obtained, crossreactivity with intact CT-B was variable and usually quite low. These antibodies characteristically required long incubation to bind CT-B in an ELISA, titered out at low serum dilutions against CT-B and did not mediate immunoprecipitation of CT-B, suggesting low affinity interactions. Consequently, these reagents proved useless in structure-function analysis. Studies assessing the ability of these anti-peptide antibodies to neutralize toxicity, block receptor recognition, or compete with the tritiated oligosaccharide moiety of GM-1 yielded consistently negative results. Binding of the antibodies to the homologous LT of porcine and human origin was marginal and difficult to interpret.

From these studies we have concluded that anti-peptide antibodies are not generally useful as a tool in structure/function analysis of these toxin proteins because of low affinity interactions. Paranthetically, it should be noted that this approach has been quite successful with certain pili peptides (Schoolnik and O'Hanley, personal communication; hence the method itself may not be flawed but is

dependent upon the system to which it is applied. Monoclonal antibodies (Mabs), however, constitute potentially high affinity reagents and have helped circumvent the difficulties we encountered.

Eleven Mabs directed against CT, eight of which recognize the B subunit, were obtained from Professor Randall Holmes. These antibodies have been used in our initial studies although Mr. Ludwig and Dr. Schoolnik have begun to prepare their own monoclonal reagents. Table 1 summarizes the binding of the monoclonal antibodies directly to CT, CT-B or to these proteins prebound to GM-1. While M5-9 and M11 recognize both CT and the GM-1/CT complex, M1-4 requires the presence of prebound GM-1. These data can be interpreted in several ways and requires further study. However, there was one unequivocal observation. M10, in contrast to all the other Mabs examined, did not bind to the GM-1/CT complex.

TABLE 1

Binding Characteristics of Holme's Monoclonal Anti-CT Antibodies

Clone	Binding		Specificity	
	GM-1/CT	CT	GM-1/CT-B	CT-B
M1	+	-	-	-
M2	+	-	-	-
M3	+	-	-	-
M4	+	-	+	-
M5	+	+	+	+
M6	+	+	+	+
M7	+	+	+	+
M8	+	+	+	+
M9	+	+	+	+
M10	-	+	-	-
M11	+	+	+	+

The observation that M10 could not recognize receptor-bound toxin suggested that this antibody's epitope resided within or near the receptor binding domain of CT-B. To explore this possibility, we conducted a competition RIA between each Mab and the oligosaccharide moiety of GM-1 (OS-GM-1). The binding of M10, and only M10, to CT-B was completely abolished by micromolar concentrations of OS-GM-1. While competition of this nature may theoretically result from gross steric hindrance or allosteric distortion, the small size of OS-GM-1 (MW approximately 800) and the preservation of every other epitope strongly implied that M10 bound amino acid residues involved in receptor recognition.

To define the amino acid sequence to which the Mabs, and M10 in particular, bound, we initially screened for crossreactivity with entries in the peptide library. Evidence for such crossreactivity could not be obtained from either direct or competitive RIAs. Since there was considerable overlap between synthetic peptides and the proteolytic

fragments, we concluded that the available Mabs probably bound non-linear or conformation- determined epitopes. Therefore, we embarked upon an alternative approach, chemical modification of the toxin, designed to alter specific amino acid residues and produce selective loss of antigenicity while preserving secondary, tertiary and quaternary structure. Preliminary data appear promising: CT-B was formylated in HCl-saturated formic acid resulting in the covalent modification of the tryptophan residue at position 88 in CT-B. This modification did not produce any gross distortion in higher order structure- formylCT-B maintains its pentameric arrangement, crossreacts with antisera against native CT-B and continues to bind six of seven monoclonal antibodies. However, formylation of toxin disrupts the binding of M10. This finding suggests that try⁸⁸ constitutes part of the combining site of M10 and, because M10 competes with the oligosaccharide moiety of GM-1 for binding to CT-B, resides within or near the receptor binding domain. The effects of other specific modifications on Mab binding are currently under study and we are currently also producing monoclonal anti-peptide antibodies and screening with native toxin for high affinity clones. By and large we are pleased with our initial venture into the use of synthetic reagents as a means to dissect the molecular basis of toxin action and we believe this experimental approach will prove to be a valuable addition to our research efforts.

2. Characterization of a *Salmonella typhimurium* adhesin

2.A. Plan of Study. In our Annual Report dated October 1983, we noted that we had begun work upon an adhesin of *Salmonella typhimurium*. There is very little information on *Salmonella* adhesins. Common or type 1 pili have been reported for several species of *Salmonella* by Duguid and his associates (8). It is widely acknowledged that representatives of the FIRN line of *S. typhimurium* which lack common pili are not any less virulent than pilated strains. However, Garth Jones and his associates (9) have reported that hemmagglutination in the presence of mannose, a phenotype felt to suggest the presence of an adhesin distinct from common pili, was correlated with the ability of *Salmonellae* to adhere to and invade HeLa cells. Our initial studies confirmed the presence of a *S. typhimurium* factor which mediated the hemagglutination of sheep and goat erythrocytes in the presence of mannose (MRHA). To date, we have examined more than 20 independent *S. typhimurium* isolates including strains of the FIRN line and derivatives of the Q1 line which lack the "cryptic" plasmid found in many *S. typhimurium* lines. All of the strains we examined exhibited the MRHA phenotype.

A brief survey of other *Salmonella* serotypes demonstrated that this phenotype was not unique to *Salmonella typhimurium* strains. Table 2 gives some of the pertinent data. It may be seen that the presence of hemagglutinating activity does not correlate with the Oantigen type.. Perhaps the most intriguing finding from these still preliminary studies was that common gastroenteritis isolates exhibited the phenotype while the hostspecific strains, more likely to cause systemic illness, were devoid of this activity.

To facilitate the characterization of the genes responsible for

the MRHA phenotype in S. typhimurium, strain SL5166 was chosen for further investigation. SL5166 is a S. typhimurium strain of TML origin which is virulent for mice ($LD_{50} = <1000$ cells), carries the "cryptic" plasmid, and was originally isolated from a human infection.

A cosmid gene library of SL5166 was constructed using cosmid vector pHC79 (10); the latter is a pBR322 derivative which contains the cos site necessary for lambda bacteriophage in-vitro packaging. Chromosomal DNA fragments of SL5166 were digested with the restriction endonuclease Sau 3A to a size of about 3045 kilobases (Kb). These were ligated into the BamHI site of pHC79 inactivating a tetracycline resistance gene. Ligated DNA molecules were packaged in-vitro and "transduced" in the E. coli K12 strain HB101. E. coli which had received recombinant molecules were selected on an appropriate growth medium containing ampicillin. The resulting colonies were screened for MRHA. A total of 5/625 clones were found to hemagglutinate sheep and goat erythrocytes in the presence of mannose. For comparative purposes we note that 2/625 strains were complemented for a proline mutation.

TABLE 2
DISTRIBUTION OF MRHA AMONG SALMONELLA STRAINS

STRAIN	NUMBER	SEROGROUP	MRHA PHENOTYPE
<u>S. typhimurium</u>	W118	B	+
<u>S. agona</u>	SL4715	B	+
<u>S. enteritidis</u>	SF1	B	+
<u>S. tilburg</u>	524/80	B	+
<u>S. austin</u>	SL5310	C1	+
<u>S. berta</u>	9/77	D1	+
<u>S. memphis</u>	SL5311	K	+
<u>S. paratyphi B</u>	48/78	B	-
<u>S. typhi</u>	253 TY	D	-
<u>S. cholerasuis</u>	SL2839	C1	-
<u>S. pullorum</u>	A468	D	-
<u>S. dublin</u>	7369	D1	-

The 5 cosmid-containing clones which showed hemagglutination were mapped by standard recombinant DNA methods using the restriction endonucleases HindIII and EcoRI. All five possessed a common 14.7 Kb DNA fragment. One cosmid clone, pHX116, was chosen for further analysis and subcloning and, subsequently, a subclone containing the 14.7 Kb fragment was isolated which could bring about hemagglutination. In addition, an E. coli carrying a subclone consisting of simply a 5.8 Kb EcoRI fragment also showed the same phenotype as the original

S.typhimurium strain. Hence, we were now able to easily examine the genetic information for MRHA as well as the adherence properties of the strains carrying the clones within a well defined genetic background.

2.8 Results and Discussion. We were interested in learning what effect the cloned hemagglutination factor might have on adherence to tissue culture cells. We had shown in considerable detail in other studies (11) that in, E. coli, the MRHA phenotype was associated with adherence to a variety of human epithelial cell lines. Using essentially these same methods, we were able to show that E. coli HB101 carrying the cloned determinants from Salmonella typhimurium cells were able to adhere to, but not invade, HeLa and Hep-2 tissue culture monolayers in the presence of alpha-methyl mannoside. HB101 containing only the cloning vector, pHC79, did not adhere appreciably to either type of epithelial cell. More recently we have been able to show that the parental S. typhimurium strain, as well as E. coli HB101 carrying the appropriate cloned sequences, can adhere to human ileal cells obtained from surgical patients. Hence, there is reason to believe that this adherence factor could play some role in the pathogenesis of Salmonella infection. In the current contract year we propose a series of experiments to directly test whether the adherence genes play such a role in experimental infection..

Efforts to gain some inkling of the receptor on the epithelial cell which is the target of the S. typhimurium adhesin have been fruitless so far. Simple approaches have not afforded any information and have included:

1). Attempts to inhibit hemagglutination by the presence of various mono- and disaccharides:

- N-acetyl glucoseamine
- xylose
- fucose
- galactose
- glucose
- rhamnose
- arabinose
- fructose
- lactose
- maltose

2.) Attempts to inhibit hemagglutination by:
antibody to Forssman antigen
treatment of erythrocytes with neuraminidase to remove sialic acid residues

3.) Attempts to demonstrate agglutination of latex beads coated with:

- beta-1,4-digalactoside
- dimannose
- Lewis A and Lewis B antigens
- Human blood groups A, B, and O

We have also moved ahead to define better the genetic organization of the genes responsible for the MRHA phenotype. HB101 containing the the subcloned 5.8 Kb. EcoRI fragment, PHX214, were infected with a variant lambda phage unable to replicate in HB101 and carrying the transposon Tn5. By using Tn5 insertional inactivation, it has been possible to more precisely identify parts of the cloned fragment essential for MRHA expression. It is of interest, we think, that Tn5 insertions which cause the loss of hemagglutination also eliminated adherence to HeLa cells. In addition by using the same minicell procedures which has served so well in the past (12) we have identified that at least four polypeptides, with molecular mass of 58, 34, 27, and 12 kilodaltons, are associated with the hemagglutination phenotype. We have not yet identified which of these proteins is the precise adhesin mediating cellular attachment. It is of some significance that all of our attempts to demonstrate pili by electron microscopy have been uniformly negative even though under the same conditions we can successfully demonstrate a variety of other pili on other enteric species including those which mediate a MRHA phenotype. The association of pili with adherence is well established in the literature of microbial pathogenicity (13). Nevertheless adherence and piliation are not necessarily synonymous. Indeed, we have just published (14) the characterization and molecular sequencing of an E. coli adherence factor operative in no less than 10% of human pyelonephritis cases which is mediated by a 16 kilodalton protein that is not a functional pilus nor, in so far as we can determine, does the molecular structure of the polypeptide show any relationship to any known pilus structure. We hope that during the coming contract year that we may define in some detail the precise structure of the adherence factor of S. typhimurium.

A genetic factor in S. typhimurium which can mediate adherence to human epithelial cells suggests it may play a role in the pathogenesis of infection. While this factor is not found in all Salmonellae, it is of some interest that the species which do not express this adhesin factor are those involved in species-specific systemic infection. Thus far, all of the commonly encountered strains of Salmonellae associated with gastroenteritis possess the factor. We wish to extend these studies. Our primary goal is to demonstrate whether the adhesin is essential for some step in the pathogenicity of S. typhimurium for the mouse. This will involve the specific construction of a strain of S. typhimurium which has a defined genetic lesion destroying the capacity of the strain to adhere. We are prepared to move ahead with the characterization of the genetic machinery involved with the synthesis and control of this adherence factor. Salmonella pathogenicity has been the subject of considerable interest but there has been relatively little study of the molecular basis for the steps involved in Salmonellosis. We believe that our work represents a useful approach to understanding better this important enteric disease.

3. Genetics and Molecular Biology of Genes of Shigellae and Yersinia which Contribute to Eucaryotic Cell Invasion and Subsequent Multiplication.

3.A Plan. Members of the Genus Shigella and Yersinia are important causes of diarrheal disease throughout the world. Shigella cause acute, usually self-limiting, diarrheal disease in humans and other primates. They are an especially important cause of mortality in developing countries (15). In contrast, the members of the genus Yersinia, Y. pestis, Y. pseudotuberculosis and Y. enterocolitica cause gastroenteritis and systemic disease in both animals and humans (16). While the Yersinia and Shigellae have a large number of differences they do share several common features in bringing about disease in mammalian hosts including the capacity to invade epithelial cells.

Early work by several investigators established that the ability to penetrate and multiply within epithelial cells was a prerequisite for virulence, although it was clear that invasion alone was insufficient to produce disease (17,18,19). Since shigellosis was restricted to primates, there was an early development of several alternative models including the HeLa cell tissue culture assay (20,21,22,23,24) and the Sereny test (25,26,27), both of which correlate reasonably well with pathogenicity. The HeLa cell assay serves as a model for invasion and intracellular multiplication. In the Sereny test, a dense inoculum of bacteria is placed under the eyelid of a guinea pig and the eye observed for the development of corneal opacity and ulceration. The invasion of corneal epithelial cells is a prerequisite for a positive Sereny test. However, it became clear that other virulence determinants must be also involved since both naturally occurring mutants and laboratory constructed mutants were found which invaded HeLa cells but failed to cause a positive Sereny test. (28,29,30,31).

It has been known for some time that virulence in Shigella and invasive strains of E. coli was lost rapidly following subculture (28). This instability has been shown to be due to the loss of plasmid-associated virulence determinants (32-36). Sansonetti and others (32-36) have shown that invasion was dependent, at least in part, upon the presence of a 140 Mdal. plasmid. Transfer of this plasmid to an E. coli strain renders the organism capable of invading HeLa cells (31). Moreover, Sansonetti and his associates were able to restore invasiveness to non-invasive isolates of Shigella flexneri by re-introduction of the 140 Mdal. plasmid. Transfer of a similar plasmid from an invasive E. coli strain to a plasmid-cured, non-invasive S. flexneri also restored the invasive phenotype. The capacity of Shigellae and invasive E. coli to translocate from the extracellular environment to an intracellular environment appears to be dependent upon a group of genes found on related, though not identical, plasmids in these species. It must be emphasized that these plasmid-mediated determinants appear to act in concert with essential chromosomal genetic information for the full expression of pathogenicity (). Yet,

the actual invasion of epithelial cells stands as one of the first essential steps in the pathogenesis of infection and the study of this phenomenon at the molecular level is a reasonable goal of our contract work in the coming years.

The pathogenic Yersiniae, like the Shigellae, harbor a plasmid which is essential for pathogenicity. However, the capacity of Yersiniae to invade epithelial cells is clearly encoded by chromosomal genes (37). The plasmid-encoded determinants of the Yersiniae appear to be involved with the biosynthesis of a distinct group of outer membrane proteins whose role in the pathogenesis of infection still remains the subject of considerable speculation and experimentation (38,39). For our current purpose, we wished only to focus upon the genetic and molecular determinants of invasiveness. With this goal in mind we began a series of experiments in invasive E. coli, Shigellae and Y. pseudotuberculosis to identify specific determinants of microbial invasion. As described in the following sections, we have succeeded in defining precise sets of genes associated with these phenotypes in both the Shigellae, invasive E. coli and Yersiniae. Moreover, it has been possible to examine, compare and contrast the contribution of these genes to epithelial cell invasion by examining gene action in the common genetic background of E. coli K-12.

3.B Results and Discussion.

3.81. Invasion determinants of Shigellae. Our initial experiments focused upon E. coli SP11 which contains a 140 Mdal. plasmid which is essential for tissue cell invasion. Using pMR5 (40), an RP1 derivative which is temperature-sensitive for replication at 37C. and contains the transposon, Tn801, conferring ampicillin resistance, the 140 Mdal virulence plasmid of SP11 was 'marked' with ampicillin resistance. This plasmid, pPCS201, was mobilized into the E. coli K-12 strain HB101 using an F^{ts} lac::Tn5 plasmid. This E. coli K-12 derivative was found to invade HeLa cells at a level comparable to the parental SP11 strain. We were satisfied that, by this criterion at least, sufficient genetic information was present upon pPCS201 to confer invasiveness upon host bacteria and we proceeded to attempt to clone these determinants using recombinant DNA methods. We constructed a cosmid bank of pPCS201 DNA using 35-40 Kb. fragments derived from Sau3a restriction endonuclease digestion of the plasmid DNA. These fragments were ligated into the cosmid vector pHC79 and packaged into lambda according to the protocol we described earlier (10). Following packaging, cosmid clones were transfected into E. coli HB101. Eight of 200 clones screened for invasion were found to have the ability to invade HeLa cells. However, these clones underwent deletion at an extraordinary rate such that we could not maintain a stable cloned line for useful analysis. Subsequently, we have attempted to employ plasmid DNA from other invasive E. coli strains and to utilize low-copy number cosmid vectors without success. We concluded that either a very large fragment of DNA was necessary for invasion which could not be stabilized in the system we utilized or that more than one contiguous genetic region was required for invasion which could not be readily accommodated within a single cosmid clone.

To better define the genetic regions present upon the pPCS201 DNA

necessary for invasion, we employed λ b221 CI857::Tn5 029amp^R and selected for insertion of the kanamycin resistance residing on the transposon into genes governing invasion by two methods. First, recent work by Maurelli (41) has shown that loss of the ability to bind the dye Congo Red (CR) can be used to screen for loss of invasion. We have confirmed this observation and, indeed, transfer of pPCS201 to the CR-negative strain HB101 resulted in the CR⁺ phenotype. Hence, following Tn5 mutagenesis, we replica plated 1000 colonies to CR kanamycin (25ug/ml.), ampicillin (100ug/ml.) plates and isolated 14 CR⁻ colonies. All of these were negative in the HeLa cell invasion assay. Restriction analysis of plasmid DNA from these mutants using the enzyme ClaI showed three classes of mutants in a 20Kb., a 15Kb., or a 7.5 Kb. ClaI fragment. While these fragments appear to be contiguous, it is still not clear whether all of these segments are required for invasion. It is possible they are not and that our findings reflect the presence of at least three distinct regions required for the invasion phenotype. Given the rather large size of the combined fragments that are required for the invasor phenotype, about 42.5 Kb., and the size limitation of the cosmid cloning procedure to 35-40 Kb. DNA fragments, it seems likely that our difficulty in isolating and maintaining a cosmid clone is a function of size limits. The same results have been observed by Sansonetti (personal communication).

Since the biochemical basis of the CR positive phenotype is unknown, we were concerned that our sample of negative mutants might be biased. We used an alternative method; therefore. A second set of Tn5⁻ mutants were isolated by mobilizing pPCS201::Tn5 mutants into a non-invasive S. flexneri background. In this way we have isolated an additional 25 non-invasive plasmid mutations. While we have not yet completed our analysis these mutants appear to span the same genetic regions as defined by the CR phenotype.

Hale, Sansonetti and their associates (42) have established that there is extensive DNA homology among plasmids isolated from S. flexneri and invasive E. coli as well as the S. sonnei FormI plasmid. In addition we have shown that there are related plasmids in S. boydii and S. dysenteriae. Loss of the plasmid in all of these species, save one, results in the loss of the capacity to invade HeLa cells. The one exception we have found is that S. sonnei devoid of the FormI plasmid retains its capacity to invade HeLa cells. This suggested to us that at least some of the invasion determinants in this species might be encoded in chromosomal genes and that they might possess a different genetic organization than seen in the virulence plasmids. With this in mind we prepared a cosmid bank of S. sonnei PC891 devoid of the 120 Mdal. FormI plasmid into the low copy number cosmid vector, pREG153. The cosmid clones were transferred to HB101 and one clone, among 500 tested, has been found to be invasive. We are in the process of analysing this clone further.

3.82. Invasion determinants of Yersinia. There is considerable evidence to support the view that adherence to, and infection of, eucaryotic epithelial cells in culture is a temperature regulated and chromosomally mediated function rather than plasmid encoded (37). In any event, while we (37,38) have explored the plasmid-

mediated functions essential to Yersinia pathogenicity in considerable detail, it was clear that in order to examine the precise mechanism of invasiveness that we would need to examine chromosomal genes of the Yersinia and to our knowledge that had not been done previously. We felt that it would be best if we could develop and examine a library of Yersinia genes in E. coli although it was not known whether Yersinia chromosomal genes would be efficiently expressed in an E. coli host background.

Consequently, Dr. Ralph Isberg, a postdoctoral fellow in our laboratory, prepared a gene library of Yersinia pseudotuberculosis using the Sau3A partial digest method described earlier. The ligation mixture was packaged into lambda phage in vitro and used to infect E. coli K-12 HB101. Clones derived from Yersinia pseudotuberculosis were isolated that could complement the leu, proA, galK, mtlA and the recA functions of E. coli K-12. Hence we proceeded to determine whether it would be possible to clone the Y. pseudotuberculosis determinants of invasion into E. coli HB101. The average size of the Yersinia DNA cloned into pREG153 was 35 Kb. Assuming that the chromosome of Yersinia approaches that of E. coli, it would be necessary to test some 200 individual clones in order to have screened an entire representation of the Yersinia genome in the E. coli recipient cell line. It seemed worthwhile therefore to attempt to devise a rapid assay for the identification of E. coli clones which had received the invasion phenotype.

The assay which we employed utilized the HeLa cell as the selective environment. Y. pseudotuberculosis DNA was packaged and used to infect E. coli K-12. The entire mixed population developing on a solid medium selective for HB101 cells carrying cloned DNA was harvested in fresh broth and grown to the mid-log phase of growth. After washing, 10^9 bacteria from the pool were added to a confluent lawn of HeLa cells grown in a 75 cm² T-flask. The infected monolayer was incubated for 3 hours so that any of the HB101 clones might attach to and invade the monolayer. The monolayer was then washed exhaustively in buffer to remove unattached cells. Bacteria which had stably attached to the monolayer or which had invaded the monolayer were isolated following TritonX-100 lysis of the monolayer by plating on the appropriate selective medium. In subsequent experiments we have modified this method to include the addition of 20 ug./ml. gentamicin to kill extracellular bacterial cells.

In the first enrichment experiment of this type that we performed, 22 clones were isolated which survived the exhaustive washing of the monolayer. Each of these were then individually assayed in the tissue culture assay. Of these, 12 were seen to bind to HeLa cells by microscopic examination and, since they survived gentamicin treatment in subsequent HeLa cell assays, we presume that they invade the tissue culture monolayer. We show data obtained from two such clones (Table 3). As can be seen, each positive clone tested gave rise to a similar number of bacteria after gentamicin treatment, washing and subsequent TritonX-100 lysis of the monolayer.

TABLE 3

Behavior of E. coli Carrying Cloned Y. pseudotuberculosis DNA

Organism	Number of Colonies/ 2 microliter aliquot
<u>Y. pseudotuberculosis</u>	1200
<u>E. coli</u> HB101 (pREG153)	4
<u>E. coli</u> HB101 (pINVA2)	1150
<u>E. coli</u> HB101 (pINVG10)	1070

These data suggested that the presence of the cloned Yersinia sequence in the E. coli K-12 strain permitted it to invade cultured cells as efficiently as the parental Yersinia strain. This view was strengthened by comparing the kinetics of binding by ³⁵S-labeled bacteria (Figure 3). These studies demonstrated that the E. coli strain harboring the clone associated with cultured HeLa cells at virtually the identical level as the Yersinia pseudotuberculosis from which the cloned DNA was derived.

It was our belief that essential genes conferring the capacity for invasiveness had been cloned into the HB101 strain from Yersinia pseudotuberculosis. We then set about to better characterize the cloned DNA sequences responsible for this property. The entire cloned cosmid sequence encompassed approximately 38 Kb. of Yersinia DNA. Tn5 insertions were introduced into the cloned genes using the methods described earlier. A number of insertions completely inactivated both the ability of the bacteria to bind to HeLa cells as well as to invade them. These two properties can be separated since HB101 carrying the cloned sequences, like the parental Yersinia strain, will tightly bind to HeLa cells at low temperature but do not initiate invasion. Invasion per se which we define as resistance to gentamicin killing in the HeLa cell assay, occurs only at 30C. In any event, all of the insertions which affected the invasive phenotype, Inv, mapped in a single 4.6 Kb. BamHI DNA fragment. On the basis of these data, the 4.6 Kb. fragment has been subcloned into pBR325 for further studies. This relatively short segment of DNA is sufficient to confer the invasive phenotype upon E. coli HB101 host cells.

It is extraordinary in one sense that what seems a complex phenomenon such as the association of a microorganism with the surface of a cultured eucaryotic cell and the translocation of a microbial cell into the eucaryotic cell substance can be conferred upon the innocuous E. coli K-12 strain by such a relatively short segment of nucleic acid from Yersinia. A number of possible explanations come to mind although the most satisfying working hypothesis is that the cloned DNA segment encodes a molecule that modifies the surface of the microorganism in some fashion. We believe we are well positioned to pursue this. We have

established methods to examine the macromolecules encoded by cloned DNA sequences in E. coli minicells, and we have recently established methods for performing immunoelectron microscopy in our laboratory and in collaboration with Dr. Claude Garon of the Rocky Mountain Laboratory. Moreover, we have shown in previous contract years that we have the capability to determine the DNA sequence and, hence, the inferred amino acid sequence encoded by cloned genes. From these kinds of data we can often infer important domains of protein structure and function. Of course another exciting facet of the future work will revolve around the relationship of these essential virulence genes with the genetic material of other invasive microorganism as revealed by DNA-DNA hybridization.

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Figure 1

Predicted Secondary Structure of LT-B Polypeptide

Computer-assisted secondary structure of LT-B polypeptide constructed using the Chou and Fasman algorithm (15,16). The coil structure indicates an alpha-helix, the "picket fence" indicates a beta sheet. In areas where both conformations are possible the alternative form is given as a dotted structure.

The parameters for construction were:

alpha Nue Ext Min	= 1.030
beta Nue Ext Min	= 1.050
absturn Min	= 0.000100
reeturn Min	= 0.000075

This is not the only structure that can be constructed from the data but represents the most probable fit of the data to the Chou and Fasman rules. Model constructed by Stephen Hirschfeld and confirmed by S. Falkow.

Figure 2

Average Hydropathicity of St-P polypeptide calculated according to the method of Hopp and Woods (19). The hydropathicity maximum occurs of 2.100 occurs at residue 29. The default values used were:

Ala = -.500	Gln = .200
Gly = .000	Tyr = -2.300
His = -.500	Asp = 3.000
Asn = .200	Lys = 3.000
Pro = .000	Arg = 3.000
Val = -1.500	Glu = 3.000
Trp = -3.400	Leu = -1.800
Cys = -1.000	Ser = .300
Ile = -1.800	Phe = -2.500
Met = -1.300	Thr = -.400

Hydropathicity average at residue i is calculated across 6 residues, from $i - 3$ through and including $i + 2$.

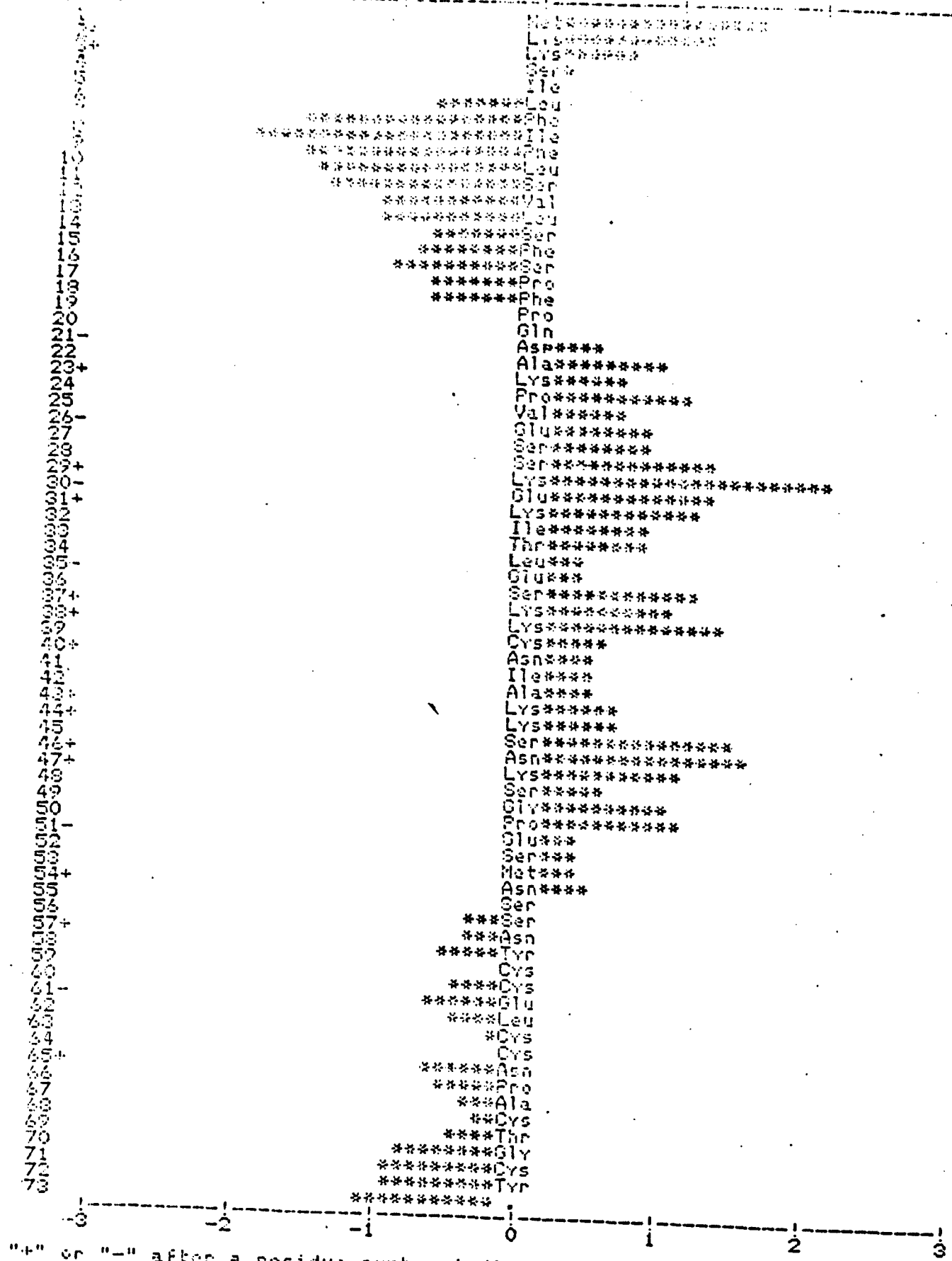


FIGURE 2

"+" or "-" after a residue number indicates a charged residue.
 The residue(s) labelled as "." in the input file
 are not included in calculating average hydropathicity values.

FIGURE 3

Kinetics of adsorption to HEp-2 cells by Y. Pseudotuberculosis and E. coli containing the plasmid that confers invasion.

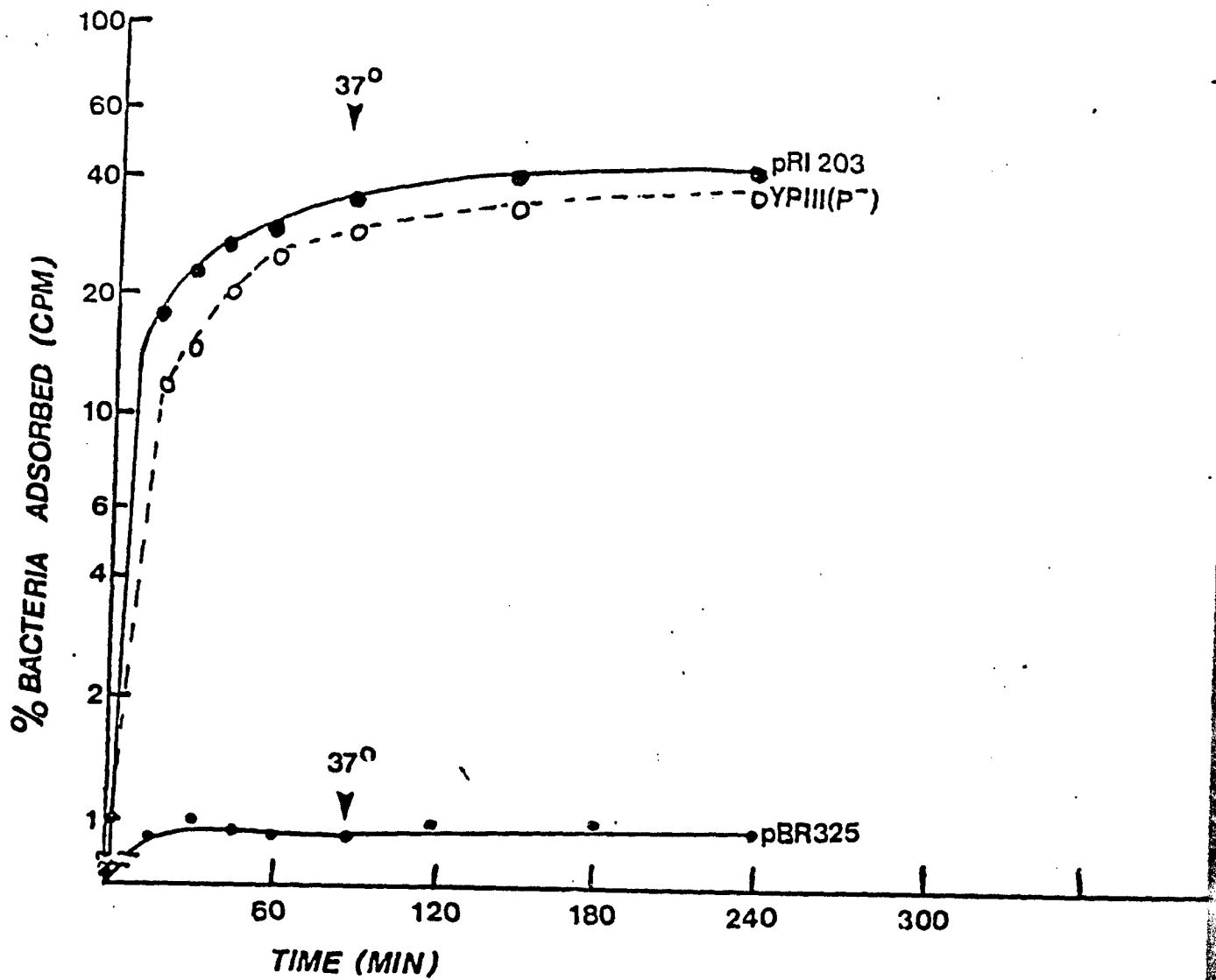
Bacteria were labeled with [³⁵S]-methionine, washed exhaustively in PBS, and chilled on ice. 1.5×10^5 CPM (about 50 μ l) of each culture was added to a series of confluent monolayers of HEp-2 cells in 1 ml vials (1×10^5 animal cells) and incubated on ice. The bacteria were pelleted onto the monolayer at 2000 X g for 15 min. at 4°C, and at various times after addition of the bacteria to the monolayers the vials were washed exhaustively with ice-cold PBS, allowed to dry, and counted for radioactivity that adhered to the monolayer. At 90 min post-infection the cultures were placed at 37°C to determine if additional adsorption would occur after raising the temperature.

($\bullet \rightarrow$ pRI203): E. coli strain HB101 harboring the plasmid that confers the invasive phenotype.

($\bullet \rightarrow$ pBR325): E. coli strain HB101 harboring the cloning vector used in this study.

($\rightarrow \rightarrow$ YPIII(P⁻)): Y. pseudotuberculosis biotype III cured of the yersinia virulence plasmid.

FIGURE 3



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